Biotechnology and Cultivation of Desert Truffles

A. Morte(✉), M. Honrubia, and A. Gutiérrez

1 Introduction

Among the ascomyces truffles, there are several genera with an excellent record as edible fungi, and two of these are of considerable economic importance: *Terfezia* and *Tuber*. Of these two genera, only *Tuber* had been cultivated commercially (for decades), until now. More recently, biotechnological methods on fungal inoculum and mycorrhizal plant production, as well as plantation management, have been developed to cultivate, for the first time, some species of the *Terfezia* genus (Honrubia et al. 2001, 2005; Morte et al. 2004, 2006). These procedures are presented in this chapter. Here, we attempt to evaluate conclusions on the basis of recent truffle production data from the first field plantations.

The genus *Terfezia* belongs to the so-called "desert truffles" which are a complex family of mycorrhizal hypogeous fungi, mainly containing species of the genera *Terfezia, Picoa, Tirmania* and *Tuber*. Their geographical distribution is limited to arid and semiarid lands, mostly in countries around the Mediterranean basin, such as: southern Spain, Portugal, Italy, France, Hungary, Turkey, from Morocco to Egypt, Israel, the Arabian Peninsula, Iran, Iraq, Libya, Syria, and Kuwait. In addition, some desert truffle species have been found in South Africa (Botswana) (Marasas and Trappe 1973), in North America and Japan (Trappe and Sundberg 1977), and China (Wang, unpublished data).

Generally, the regions where desert truffles grow have an annual rainfall which ranges from 50 to 380 mm. The truffle season produces good yields if the rainfall ranges from 70 to 120 mm in North African countries and from 100 to 350 mm in countries of southern Europe. The rainfall distribution is very important as far as both quantity and the time of the rainfall are concerned; that is, no later than the beginning of December in North African and Middle Eastern countries and no later than the beginning of October in countries of southern Europe.

A. Morte
Dpto. Biología Vegetal (Botánica), Facultad de Biología, Universidad de Murcia,
Campus de Espinardo, 30100 Murcia, Spain
e-mail: amorte@um.es

A. Varma (ed.) *Mycorrhiza*,
© Springer-Verlag Berlin Heidelberg 2008

Desert truffles are of considerable interest for ecological reasons because of the low water input required for cultivation, which makes them an alternative agricultural crop in arid and semiarid areas, and one of commercial interest because of the prices they fetch in the open market. Part of the mystique of truffles is, of course, their often extravagant cost. Desert truffles are cousins of the white fragrant Italian (*Tuber magnatum*) and Perigord (*Tuber nigrum*) truffles. However, the prices of the desert truffles are much lower than those of these *Tuber* species. One of the reasons is that desert truffles are not as strongly flavored as the *Tuber* species. It is probably a question of good marketing and presenting them in an attractive, glamorous way to people.

As desert truffles grow much more prolifically than *Tuber* species in general, they can be used in a much greater volume. Sizable quantities of several species of wild *Terfezia* are collected and marketed in southern Europe, parts of North Africa, and other countries bordering the Mediterranean. However, natural areas of desert truffles have been disappearing in the last 50 years. Large areas of the coastal desert in Egypt and Libya were mined in World War II. More recently, in Kuwait, certain aspects of the 1990–1991 Gulf War seem to have ruined many truffle-gathering areas. In Europe, this has been due to the huge building construction process taking place in these "sunny" areas over the last 5 years.

2 Chemical Composition

Desert truffles have been known to be edible for 3,000 years (Chang and Hayes 1978). Several studies on their chemical composition have shown that they are rich in proteins, amino acids, fiber, minerals and carbohydrates (Ackerman et al. 1975; Al-Delaimy 1977; Ahmed et al. 1981; Bokhary et al. 1987, 1989; Bokhary and Parvez 1993; Murcia et al. 2003).

The protein content, which averages 20% of the dry weight in desert truffles, is significantly higher than in most vegetables and other fungi. One 250-g serving of desert truffle can contribute 23–27% of the recommended daily intake of protein. These truffles could be cultivated in several of the developing countries of Africa and the Near East, making them important sources of protein for human consumption (Murcia et al. 2003). Also, the National Cancer Institute recommends that the daily diet contains between 25 g and 35 g of dietary fiber (Wu et al. 1994), values that are much higher than the average level actually consumed. Accordingly, one 250-g serving of truffles could contribute 16–22% of the recommended daily intake of fiber.

Our results on *Terfezia claveryi* (Fig. 1a, b) and *Picoa lefebvrei* showed good levels of fiber and monounsaturated fatty acids and few changes in the proximate composition of truffles during industrial processing (freezing and canning). As a consequence, the consumption of these processed truffles is recommended (Murcia et al. 2003).

One of the most interesting food properties of desert truffles is their antioxidant activity with regard to their ability to inhibit lipid oxidation (Murcia et al. 2002). *T. claveryi* and *P. lefebvrei* have higher percentages of oxidation inhibition than some common food antioxidants, like α-tocopherol, BHA (E-320), BHT (E-321) and propel

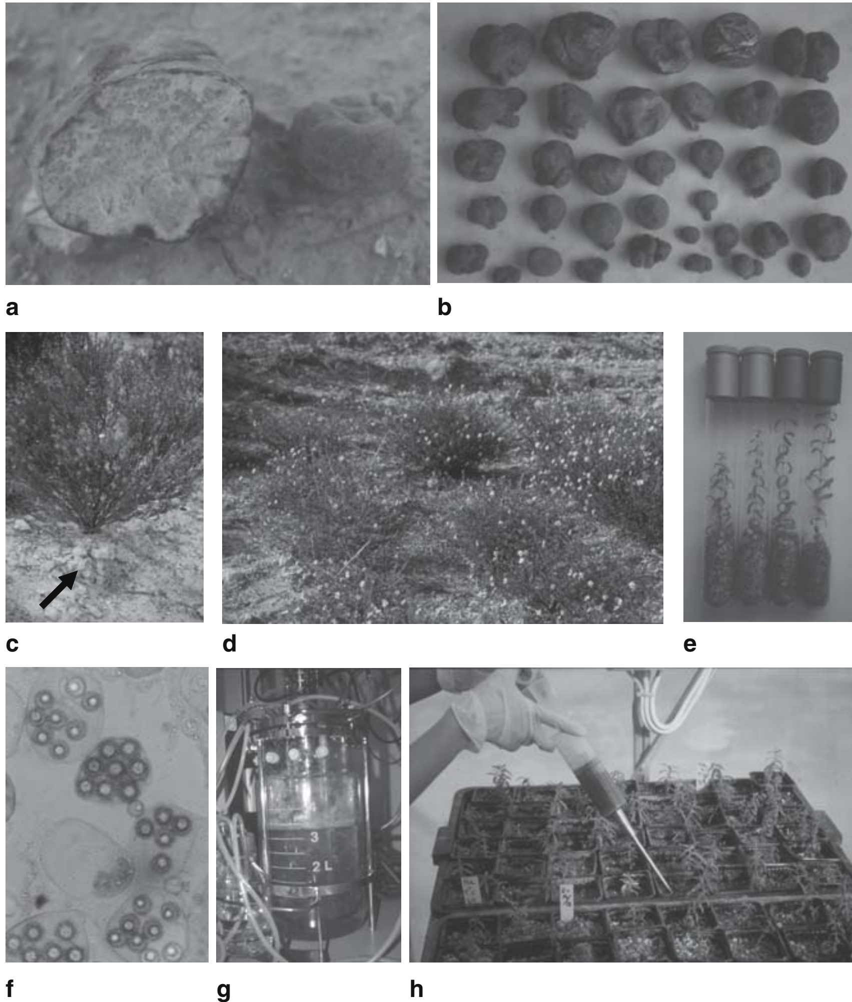

Fig. 1 **a,b** Ascocarps of *Terfezia claveryi*. **c** The host plant *Helianthemum almeriense* with a crack (*arrow*) in the nearby soil which indicates the presence of a truffle. **d** Plantation 1 of *H. almeriense* × *T. claveryi* in Lorca (Murcia, Spain). **e** In vitro mycorrhizal *H. almeriense* plants on vermiculite watered with the MH medium. **f** Asci and mature ascospores of *T. claveryi*. **g** Fermentor with a *T. olbiensis* mycelium culture. **h** In vivo inoculation of *H. almeriense* with a *T. claveryi* spore solution in the nursery

gallate (E-310), even after being subjected to the industrial freezing and canning processes (Murcia et al. 2002). More specifically, canned truffles present significant losses of antioxidant activity, while the frozen products suffer less extensive losses. Raw truffles exhibit a strong antioxidant activity as scavengers of several oxygen species. This finding supports the replacement of synthetic antioxidants with natural truffle extracts. Thus, raw and frozen truffles can be considered promising candidates for

industrial processing, thus permitting their year-round consumption. Furthermore, they can be considered as functional foods (Medina et al. 1999).

Despite their interesting chemical composition and antioxidant activity, a promising antibiotic activity has been detected in desert truffles (Rougieux 1963; Chellal and Lukasova 1995; Janakat et al. 2004, 2005). All these findings make desert truffles an interesting crop to be cultivated.

3 Biotechnological Aspects

Most desert truffles establish mycorrhizal symbiosis with species of the *Helianthemum* genus (relatives of the common rock rose cultivars) from the *Cistaceae* family. The fruiting bodies or truffles are often found nearby. They slightly push up the overlying soil, cracking it (Fig. 1c), and such cracks are used to help find the fungus. A screwdriver is generally used to push them up.

Their ecology changes depending on the host plant species. We can find the same truffle species, for example *Terfezia claveryi*, with the perennial species of *Helianthemum almeriense* (Fig. 1c) in an open low scrubland dominated by woody bushes. Alternatively, there are the same fungal species with *Helianthemum ledifolium*, annual, and *Helianthemum canariense*, perennial, in xerophytic meadows. The soil characteristics are also of a wide range with regard to texture (from sandy to calcareous), pH (acid and alkaline) and the rates values of electrical conductivity, organic carbon and the C/N rate.

The results presented in this chapter refer to three species of desert truffles: *T. claveryi*, *T. olbiensis* and *Picoa lefebvrei*. They are the most frequently found desert truffles in alkaline and calcareous soils from the Region of Murcia, in southeast Spain.

All these considerations should be taken into account in order to produce mycorrhizal plants suitable for different field conditions.

3.1 Plant Production

In order to establish mycorrhizal desert truffle plants in the field, it makes more sense to use perennial plant species than annual ones to maintain this culture for a minimum of 10 years. Accordingly, two perennial *Helianthemum* species have been selected for this purpose.

Helianthemum almeriense is a shrub that is 10–20 cm tall, whose distribution is restricted to southeast Spain and the north of Africa (Morocco). It appears in open places, in dry, stony, limestone, mica, marl, or marl with gypsum soils, even in sandy terrains, at an altitude of between 0 and 500 m (López-González 1993). *Helianthemum violaceum* has similar botanical characteristics but it colonizes a larger territory than *H. almeriense*, not only in Spain but also in southern Europe and North Africa.

The germination of their seeds is usually erratic. It is necessary to scarify the seeds for good germination results. Moreover, 50% of young seedlings normally die after 4–6 weeks in pots during nursery production. For these reasons, micropropagation of these plant species is an interesting option, not only as a variant of the traditional propagation method, but also to be able to control and better understand the nutritional requirements, thus enabling good mycorrhization (Morte and Honrubia 1997).

According to Morte and Honrubia (1992, 1997) and Zamora et al. (2006), micropropagation protocols mainly consist of:

1. Initial explants: shoot tips and nodal segments of in vitro germinated seeds.
2. Culture conditions: 22 ± 2°C, 40 μmol/m^2/s Grow lux fluorescent light and 16-h photoperiod.
3. Culture medium: Murashige and Skoog (MS) (1962), with 0.8% Panreac-agar, 3% sucrose, pH 5.8.
4. Multiplication stage: MS medium with 0.46 μM kinetin (*H. almeriense*) or 0.023 μM kinetin (*H. violaceum*) for 4 weeks. The propagation system followed with these species was principally the formation of axillary buds and the culture of nodal segments. To a greater extent, the incorporation of kinetin to the medium caused the elongation of the explants, which allows them to be cut into several nodal segments. Kinetin was more effective than the other cytokinins tested (BA and 2iP) in the production of shoots. The multiplication rate was 7.72 shoots per initial explant for *H. almeriense* and 2.55 for *H. violaceum*.
5. Elongation stage: It was not necessary to change the culture medium for shoot elongation as the shoots elongated in the same multiplication medium. This was due to the propagation system used: the formation of nodal segments, which resulted in explants of up to 10 or 11 cm long at the multiplication stage. At the end of the multiplication stage on the other hand, the type of cytokinin used, and the relative low concentration, allowed the axillar-ramification formed shoots to reach an adequate length (2.5–3 cm) for their direct use at the following rooting stage. In *H. almeriense* and *H. violaceum*, therefore, the shoot multiplication rate is directly related to shoot elongation and the number of nodal explants available in each species.
6. Rooting stage: Spontaneous rooting was observed during the simultaneous multiplication and elongation stages in cultures with a low level of cytokinins, which varied between 25 and 80% for both plant species. This rooting percentage was improved to 100% with 1/4 macronutrient MS dilution for *H. almeriense*, and full MS for *H. violaceum*, and both without plant growth regulators for 3 weeks.
7. Weaning stage: The rooted plantlets were potted in a peat-sand-vermiculite mixture (3:1:1, v/v) and gradually exposed to reduced relative humidity for 2–3 weeks in the greenhouse. At the end of 1 month, approximately 95% of the plantlets survived.

In summary, the *in vitro* plant propagation of these *Helianthemum* species takes 10 weeks in total. It is a quick micropropagation protocol because plant multiplication, elongation and rooting may occur at the same subculture and, consequently, it is also a cheap protocol because of the small amount of plant growth regulators and manual labor required.

The *Helianthemum* plant production obtained directly from seed germination under nursery conditions takes at least 6 months to obtain plants suitable for fungal inoculation. This traditional propagation method is cheaper than in vitro propagation. However, both methods can be used for plant production, although it is advisable to use micropropagation if an in vitro culture laboratory is available.

3.2 Desert Truffle Inoculum Production

Two types of fungal inoculum have been used successfully to produce desert truffle mycorrhizal plants: spores and mycelium.

Spores from the *Terfezia* and *Picoa* species should be mature in order to facilitate their germination (Fig. 1f). The main problem found when using this type of inoculum is that the colleted ascocarps are immature for most of the time. We have observed that keeping the ascocarps covered by soil at 4°C for 10–15 days could induce spore maturation. The ascocarps with mature spores are then cut into pieces and dried at room temperature or in an oven at 60°C for 2 days. The dried ascocarp pieces are scratched and kept in glass jars at room temperature and in darkness until used. These spores are able to germinate even after two years in storage.

The spore suspension is performed taking into account the maturation of the spores. The spore suspension from well mature ascocarps consists of 10 g of scratched ascocarps per liter of distilled water. This spore solution is shaken overnight (12 h) before the inoculation of the plants. Each plant is inoculated with 5 ml of the spore solution, with a final spore concentration of 10^5–10^6 spores per plant. The spore germination is around 65% when the spores are from mature ascocarps.

The mycelium isolated from the desert truffles normally grows quite slowly, particularly during the first year in culture from the in vitro establishment. The best growth media are MMN (modified Melin-Norkrans) agar medium and PDA (potato dextrose agar) medium. pH should be adjusted at 7.0 if the ascocarps are from alkaline calcareous soils. Desert truffle mycelium can be used directly from the plates as inoculum for in vitro mycorrhizal synthesis (Morte et al. 1994; Morte and Honrubia 1995, 1997), and from liquid fermentation for both in vitro and in vivo inoculations.

Some ectomycorrhizal fungi can be grown on an industrial scale using liquid medium which is stirred and/or supplied with air within a large microbial culture vessel, called a bioreactor or fermentor (Fig. 1g). The fermentor culture has only been successful with the species *T. olbiensis* strain 111ET. The production of *T. olbiensis* mycelial inoculum was carried out in MMN liquid medium with a Braun BIOSTAT® B 5-l fermentor. A started inoculum is first prepared in a 250-ml flask with MMN liquid medium at pH 7.0 and grown for 20 days, then transferred to a 5-l fermentor for a 36-day period. During growth in the fermentor, the temperature is maintained at 23°C, the aeration rate is 1.5 l/min, the rotation speed is 100 *g*, with 60% dissolved oxygen. The pH of MMN medium is fixed at 7.0 and maintained during the fermentation process by adding 0.5 N NaOH when required. These conditions are set up at the beginning of the fermentation process and automatically

monitored by the bioreactor. Periodically, the stir speed is elevated to 1,000 *g* to break the mycelium and so to stimulate its growth.

During the fermentation process, *T. olbiensis* mycelium presents a 15-day acclimatization phase, in which there is no mycelium growth. After this, the mycelium grows in a sigmoid manner in parallel with a pH, and pO_2 decreases until reaching an exponential phase from day 29. At the end of the fermentation, the dry weight of mycelium is 1.16 g of mycelium per liter of medium.

These results show that the fungal growth in the bioreactor is much faster and more effective in terms of biomass production than the cultivation of fungi in flasks by traditional means (Le Tacon et al. 1985; Carrillo et al. 2004).

Finally, after 30 days of storage at 4°C, the fermentor-produced mycelium was used for mycorrhization trials and was able to infect *H. almeriense* plants as usual.

3.3 Mycorrhizal synthesis

The mycorrhizal synthesis between desert truffles and the *Helianthemum* species is carried out in different ways according to the type of fungal inoculum (spores or mycelium), the plant source (seedlings or micropropagated plantlets) and the culture conditions (in vivo or in vitro) used (Fig. 2).

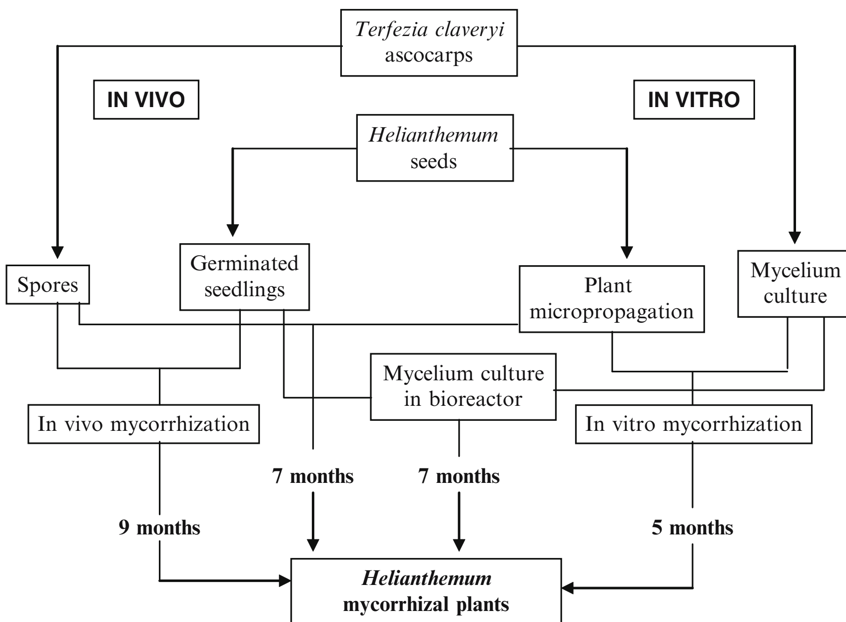

Fig. 2 In vivo and in vitro ways of producing desert truffle mycorrhizal plants and the time required for each of them

3.3.1 In vitro mycorrhization

In the in vitro system, when micropropagated *Helianthemum* plants and the pieces of agar with mycelium of *T. claveryi* or *P. lefebvrei* are used, is the quickest way (5 months in total) to produce mycorrhizal plants (Fig. 2). This synthesis is carried out on MH medium (Morte and Honrubia 1994, 1995), especially designed for this purpose, although it has been also obtained on MMN medium (Morte et al. 1994) both at pH 7.0 and solidified with agar. The rooted plantlets obtained in the MS medium are inoculated with two pieces of agar, $0.25\,cm^2$ in surface, with mycelium per tube. The tubes have a diameter of 2.5 cm and are 20 cm long. Twenty-five ml of medium are normally used per tube. The mycelium grows from the surface of the agar pieces, which serve as an inoculum, towards the interior of the culture medium in the test tube, within a 2-week period. This mycelial growth into the agar of the tube allows the mycelium to colonize the entire root system of the plantlets. The mycorrhization percentage obtained varies from 61 to 75% after 2 months in culture. This percentage is very similar for the synthesis obtained on both the MMN medium (Morte et al. 1994) and MH medium (Morte and Honrubia 1995). In contrast to MMN mycorrhization in the MH medium, however, there are no problems with the in vitro survival during the time that the rooted plantlets take to mycorrhize. This could be due to the fact that its composition is more similar to that of the MS micropropagation medium and has a greater number of nutrients than the MMN medium (Morte and Honrubia 1994).

Although the use of agar as a substrate for in vitro synthesis has given good mycorrhization percentages, sterilized vermiculite watered with MH liquid medium is strongly recommended for a semi-large-scale mycorrhizal plant production because it is more easily manageable than other types of substrate to transfer plants from in vitro to in vivo conditions (Morte et al. 2000; Gutiérrez 2001). Vermiculite also enables us to observe the growth of the mycelium on the root, and thus the start of mycorrhizal formation (Fig. 1e).

The mycorrhization percentages among the *Helianthemum* species (*H. almeriense, H. viscarium* and *H. violaceum*) and *T. claveryi* or *P. lefebvrei*, in in vitro conditions vary from 30 to 50% at 4 weeks, 60 to 75% at 8 weeks and 75 to 100% at 12 weeks from inoculation, assessed on cleared and stained root samples (Phillips and Hayman 1970), and estimated according to the gridline intersect method (Giovannetti and Mosse 1980). To optimise the best harvest time for these in vitro mycorrhizal plants, the amount of active mycelium in mycorrhizal roots has been estimated using the ergosterol measurement by HPLC at 280 nm, according to Gutiérrez et al. (2001). The ergosterol content in the mycorrhiza formed was analyzed at 4, 8 and 12 weeks after inoculation. The ergosterol present in the mycorrhizal roots of *H. viscarium* is less abundant than in the mycorrhizal roots in *H. almeriense*. In both the *Helianthemum* species, the ergosterol concentrations at 4 weeks after inoculation were 0.157 and 0.021 µg/mg of the fresh weight (FW) for *H. almeriense* and *H. viscarium*, respectively. These levels increase considerably at 8 weeks (0.214 and 0.163 µg/mg FW) and decrease markedly at 12 weeks (0.182

and 0.024 μg/mg FW). According to these data, the mycelium activity at 2 months is greater than that obtained at 3 months, even though mycorrhizal formation is slightly lower at 2 months than at 3 months. For this reason, we consider that 2 months is the optimum time to harvest in vitro mycorrhizal plants and transfer them to greenhouse conditions (Gutiérrez et al. 2001).

To transfer the in vitro mycorrhizal plants to ex vitro conditions, a mixture of sterilized soil, vermiculite 8:1 (v/v), is used. The soil is collected from areas where *Helianthemum* species grow naturally.

The advantages of this production system are that it is quick and nonseasonable because the mycelial inoculum and the micropropagated plants can be produced whenever required. However, an in vitro culture laboratory and specialized personnel are required which could elevate the final cost of the mycorrhizal plants.

3.3.2 In vivo Mycorrhization

Mycorrhizal plants are also obtained in nursery conditions directly from germinated seeds or from acclimatized micropropagated plants.

When seedlings are used, they must be 5–6 months old before fungal inoculation. Before this age, *Helianthemum* seedlings are too small and their root system is not properly developed and suitable for mycorrhization. These 6-months to year-old seedlings are inoculated with desert truffle spore suspension and it is necessary to wait 3 months to obtain good mycorrhizal plants. Spore suspension is directly applied close to the roots with a large needle connected to a syringe with the spore suspension (Fig. 1h). Nine months in total are required, this being the longest time spent to obtain our purpose (Fig. 2).

Two options exist to shorten this time: (1) the inoculation of acclimatized micropropagated plants with the spore suspension, and (2) the inoculation of seedlings with mycelial suspension obtained from fermentation in a bioreactor. In both cases, the time is reduced to 7 months. In the former case, the 2-months reduction is due to the quick plant micropropagation. When the micropropagated plants are acclimatized at 4 months, they have a good root system suitable for mycorrhization. However, 3 months are necessary for spores to colonize the *Helianthemum* roots well. In the second option, the saving in time is caused by the fast root colonization by the mycelium (only 1.5–2 months).

In short, the four options are designed to obtain a continuous desert truffle mycorrhizal plant production and to also avoid any limiting factor in this production.

3.4 Mycorrhiza Characterization

The characterization of the mycorrhiza formed in the *Helianthemum* root systems by these desert truffles is extremely important to assure high-quality produced

mycorrhizal plants. Furthermore, the maintenance of the desert truffle mycorrhiza should be checked in the root system after planting the mycorrhizal plants in the field, at least once a year. For this reason, the morphological and molecular characterization of the desert truffle mycorrhiza has been carried out.

3.4.1 Morphological Characterization

Under the stereomicroscope, four different mycorrhizal systems are observed in *H. almeriense* from different types of samples (naturally colonized roots, mycorrhiza formed in pots and in vitro-formed mycorrhiza): a club-shaped mycorrhiza, a capitate mycorrhiza, a moniliform mycorrhiza and a branched mycorriza (Gutiérrez et al. 2003). The most frequent type of these mycorrhizal systems, which appears in 46% of the studied plants, is the club-shaped mycorrhiza (Fig. 3a), an unbranched mycorrhiza, 6–8 µm in length. It has a whitish-ochre enlarged apex (0.6 µm) and a brownish-to-ochre constricted base of varying length. In general, external mycelium is observed around all the mycorrhizal systems. This mycelium is loose, bright white and not organized into a true sheath except in the in vitro mycorrhiza of both the fungal species used (*T. claveryi* and *P. lefebvrei*), where a true sheath is observed. The frequency of appearance of each mycorrhizal system neither changes with the different seasons nor with the mycorrhizal culture conditions of the fungal species used.

The four mycorrhizal systems present the same type of development at the cellular level, which only changes with the synthesis conditions but not with the fungal species. In field conditions, root colonization is mainly intracellular (Fig. 3b), forming an endomycorrhiza with large, septate and moniliform hyphae which surround the host nucleus that is frequently in a central position (Fig. 3b). Colonization only concerns the cortical cells of the roots. Intercellular hyphae are rarely observed. In no case is a sheath observed around the root; only in some cases do isolated hyphae appear around the roots (Gutiérrez et al. 2003). However, an ectomycorrhiza and ectendomycorrhiza without a sheath are formed in mycorrhiza synthesized with both desert truffles in pots under greenhouse conditions (Fig. 3c), similar to those described in *H. salicifolium* and *T. claveryi* mycorrhiza (Dexheimer et al. 1985). In the end, colonization is also intercellular in in vitro conditions, like a typical Hartig net, and only occasional hyphae are observed inside the cells (Fig. 3d). Groups of hyphae are frequently found between cortical cells, causing an important dilation of the intercellular spaces so that these cells show an elongated form. However, the main characteristic of the mycorrhiza formed *in vitro* is the presence of a well developed sheath, 50 µm thick (Fig. 3d). This sheath formation could be due to the vigorous mycelium growth on the MH mineral medium, which contains sucrose as a carbon source, and also result from the small physical space inside the test tubes which would force hyphae to surround the roots formed in vitro. Since the phosphorus content of the soil used for the mycorrhiza synthesized in pots was almost half (28.7 ppm) of that used for the in vitro synthesis in the MH medium (42.5 ppm), this may have influenced the type of mycorrhiza formed, although other factors varying between the experimental conditions could have contributed to this phenomenon

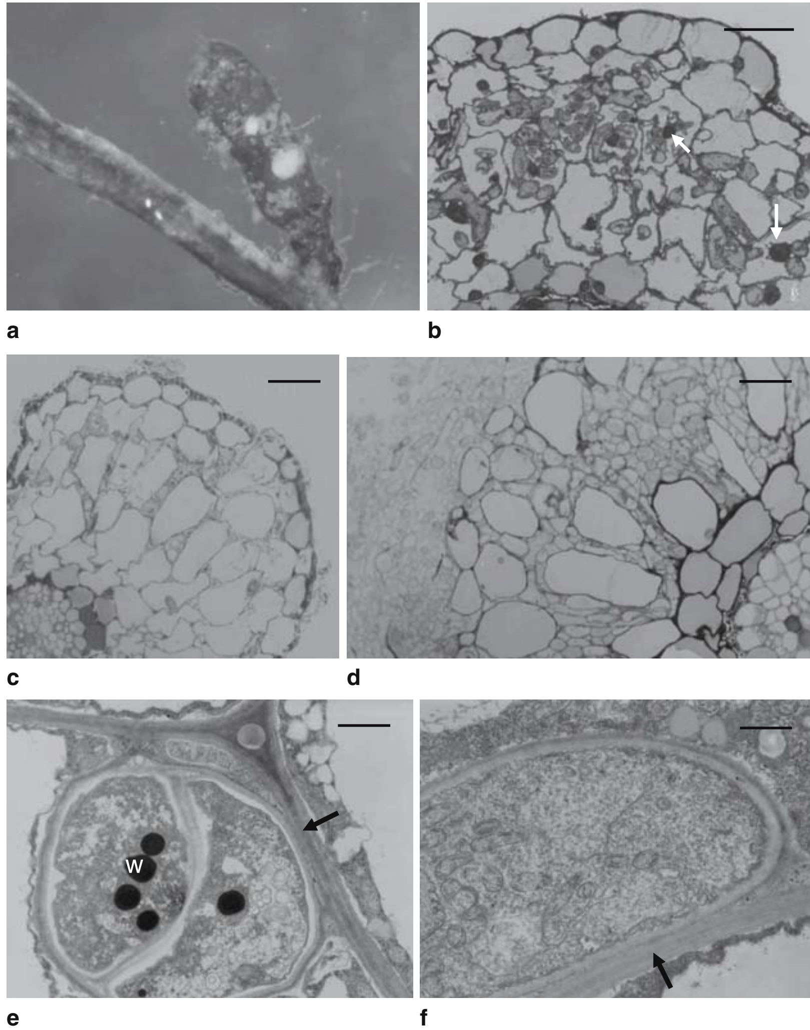

Fig. 3 **a** The club-shaped mycorrhiza in H. almeriense roots. **b** A mycorrhizal root of *H. almeriense* collected from field areas; colonization is intracellular. The nucleus of the host cell is in a central position surrounded by fungal hyphae (*arrows*). **c** Mycorrhizal roots of *H. almeriense* and *T. claveryi* in greenhouse conditions, forming an ectomycorrhiza and ectendomycorrhiza without a sheath. **d** Mycorrhizal root of *H. almeriense* synthesized under in vitro conditions with *T. claveryi* mycelium, with intercellular colonization and a sheath. **e,f** Intracellular hyphae with a host cell wall–fungal wall interface (*arrows*) and Woronin bodies (*w*) close to the septum. **b,c,d** *bars* 40 μm, **e** *bar* 0.35 μm, **f** *bar* 0.5 μm

(Gutiérrez et al. 2003). These data obtained for *H. almeriense* support those obtained for the *H. guttatum* and *Terfezia* and *Tirmania* species whose substrate fertility affects mycorrhiza morphology (Fortas and Chevalier 1992).

In conclusion, culture conditions can induce changes in mycorrhiza morphology and there is no clear barrier between the two main types of mycorrhiza organization in the *Helianthemum* species (Gutiérrez et al. 2003). Recently, two genes from *T. boudieri* (GenBank DV205797, DV205787) have been associated with ectomycorrhizas under pre-infection growth conditions, but with endomycorrhizas under mycorrhizal conditions. These genes could be involved in determining the type of association to be formed (Zaretsky et al. 2006).

Three ultrastructural studies on the mycorrhiza formed by *T. claveryi* with different *Helianthemum* species have confirmed the regular presence of *T. claveryi* intracellular hyphae in direct contact with the host plant wall (Fig. 3e, f), a localization which seems to be characteristic of the *T. claveryi* mycorrhiza organization (Dexheimer et al. 1985; Fortas 1990; Gutiérrez et al. 2003).

3.4.2 Molecular characterization

In order to identify different species of desert truffles inside the roots, DNA was extracted from 3–20 mg of ascocarps from different desert truffles species, their axenic mycelia and the mycorrhiza formed by *H. almeriense* x *T. claveryi*, according to the method described by Gutiérrez et al. (1995). The ITS region was successfully amplified by RFLP analysis. The ITS amplification product is a single band of 600 bp in size for all the isolates of *T. claveryi* as well as for the other studied species (*T. boudieri, T. olbiensis, T. arenaria* and *P. lefebvrei*) and for the mycorrhiza. It is possible to differentiate all these fungal species not only by a combination of the ITS region (without restriction), but also from the different patterns observed after treating the PCR fragments with the *Alu* I, *Hinf* I and *Msp* I restriction enzymes. Some of these ITS sequences of the rDNA from *T. claveryi, T. olbiensis* and *P. lefebvrei* are deposited in the GenBank.

The mycorrhiza ITS region restriction was performed with the enzymes *Alu* I and *Hinf* I. Restriction with *Alu* I gives two fragments of the same size than those obtained for *T. claveryi* (400 and 275 bp), and restriction with *Hinf* I gives two fragments of the same size than those also obtained for *T. claveryi* (300 bp).

4 Cultivation of the Desert Truffle *Terfezia claveryi*: Plantation Establishment and Management

An ecophysiological study on the mycorrhizal association *H. almeriense-T. claveryi* demonstrates that the water potential, transpiration, stomatal conductance and net photosynthesis are higher in mycorrhizal plants than in nonmycorrhizal plants. Moreover, this increase is higher in water-stressed plants than in well watered plants (Morte et al. 2000). Drought stress tolerance could be partly attributed to specific physiological mechanisms on chlorophyll content and gas exchange induced by the presence of *T. claveryi*. This means that the low water input required for cultivation makes them an alternative agricultural crop in arid and semiarid areas.

Furthermore, the introduction of desert truffles into dry environments may be a useful way to rehabilitate lands which have been considered unproductive to date. Both the productive nursery and agriculture sectors of nonirrigated land will be favored by the possibility of installing an alternative cultivation in what have been unproductive lands to date. They are products that will improve not only the quality of the land, avoiding soil erosion, but also the economy of social and economically depressed areas, and will add value to these territories.

Cultivation of desert truffles has been hampered by the difficulty of obtaining a good pure mycelium growth and good spore germination. Despite these difficulties, many mycorrhizal symbioses have been obtained between desert truffles and the *Helianthemum* species in greenhouses (Awameh et al. 1979; Cano et al. 1991; Chevalier et al. 1984; Gutiérrez 2001) and in in vitro conditions (Roth-Bejerano et al. 1990; Fortas and Chevalier 1992; Kagan-Zur et al. 1994; Morte et al. 1994). However, no plantations with mycorrhizal plants have been carried out to date.

Until the present time, eight desert truffle plantations have been organized in Spain (Table 1) using *H. almeriense* as a host plant mycorrhized with *T. claveryi*. These mycorrhizal plants were obtained in both nursery and *in vitro* conditions (see Section 3.3). Six of these plantations have been set up in the Region of Murcia, southeast Spain, at an altitude of 600 m. The first *T. claveryi* plantation was made up of 60 *H. almeriense* mycorrhizal plants in May 1999 at Zarzadilla de Totana (Lorca, Murcia, Spain) (Fig. 1d; Table 1). The last two new plantations were set up in spring 2006 in Andalusia,

Table 1 *Terfezia claveryi* production in the different plantations in Spain. (Data obtained up to September 2006)

Plantation	Place (Province)	No. of mycorrhizal plants	Plantation date	Management	Truffle production (kg/ha)		
					1999– 2004	2005	2006
1	Lorca (Murcia)	60	May 1999	Yes	1,147.6	0.6	1.6
2	Lorca (Murcia)	188	July 2001	No	4.8	-	-
3	Lorca (Murcia)	83	March 2002	No	19.5	0.02	-
4	Lorca (Murcia)	196	March 2002	Yes	29.4	600	120
5	Mula (Murcia)	80	March 2002	No	8.2	-	-
6	Lorca (Murcia)	640	January 2003	No	-	-	-
7	Sierra de María (Almería)	2,000	April 2006	In process	-	-	-
8	Chirivel (Almería)	3,000	April 2006	In process	-	-	-

specifically in the province of Almería, with 2,000 and 3,000 plants, respectively, as part of the Cussta Programme from the regional government of Andalusia.

In general, soils used for plantations are characterized by a clay-loamy texture, basic pH (8.5) and low rate values of electrical conductivity (123 µS/cm), organic carbon (0.9–3.9) and C/N rate (7–10). These characteristics, above all pH and texture, are different for other *Terfezia* species such as *T. arenaria* and *T. leptoderma* in Kuwait (Awameh and Alsheik 1978), Morocco (Khabar 1988) or Spain (Moreno et al. 1986), *T. pfeilli* in Botswana (Taylor et al. 1995) and *T. terfezioides* in Hungary (Bratek et al. 1996; Kovacs et al. 2003) which normally grow in acid and sandy soils.

The first cultivated desert truffles were obtained in April 2001, 23 months after the planting date, in plantation 1. Presently, this time has been reduced to 12 months with the adequate agricultural management.

We have developed a very simple management plantation protocol, after the experience acquired over the years, and after reducing the fructification time from the plantation from 23 months to 12 months (data obtained from plantations number 1 and 4). In short, it consists of mainly planting in spring, one single irrigation during the dry summer, at the end of August, of around 60–100 l/m^2, and a yearly weeding after the third year of plantation.

After testing in all seasons of the year, spring has been selected as the best time to set up a plantation owing to its moderate temperatures, the abundance of precipitation and the long photoperiod. The rainfall dependence of desert truffle fruiting is one of the most influential factors to be considered for a successful cultivation. In the region of Murcia, after years with a rainfall of between 350 and 400 mm, the estimated desert truffle production in natural areas varied between 50 and 170 kg Ha^{-1} (Honrubia et al. 2003). An irrigation system in the plantation is not necessary when the rainfall is available because the mycorrhizal association is well adapted to arid and semiarid climates (Morte et al. 2000). However, irrigation should be applied in dry years when the rainfall is less than 150 mm, at the end of the summer (August/September) and a second one at the beginning of the fruiting season (January/February) in very dry years. A drip irrigation system is recommended to save large amounts of water, although watering by sprinklers is also effective. The amount of water applied could range between 60 and 100 l/m^2 depending on the plant status. In the end, one manual yearly weeding is necessary, particularly after the third year of the plantation, in order to avoid plant competition for water and to maintain the open and sunny desert truffle ecosystem. A superficial weeding with a weeding hoe is recommended every 3 or 4 years in the same reason. To facilitate this management, a plantation frame of 3 × 3 or 3 × 2 m should be used.

The application of this plantation management is necessary to maintain the desert truffle production over time because without it, plantations have lost their productivity after 2 and 3 years (Table 1). Moreover, this management is essential during years of severe drought, like 2005 and 2006 in southeast Spain (Table 1). However, the desert truffle production fluctuates from one year to another in the same plantation, despite the management. These fluctuations could be due to other environmental or soil conditions, such as temperature and relative humidity, which always influence any crop production in the field.

Acknowledgements This work was supported by the projects CGL2007-61175/BOS (Ministerio de Educación y Ciencia, Spain) and 03121/PI/05 (Fundación Séneca, Murcia, Spain).

References

Ackerman LGJ, Vanwyk PJ, Du Plassis LM (1975) Some aspects of the composition of the Kalhari truffle of N'abba. South Afr Food Rev 2:145–147

Ahmed AA, Mohammed MA, Hami MA (1981) Libyan truffles "*Terfezia boudieri* Chatin". Chemical composition and toxicity. J Food Sci 11:145–147

Al-Delaimy KS (1977) Protein and amino acid composition of truffles. Can Inst Food Sci Technol J 10:221–222

Awameh M, Alsheikh A (1978) Laboratory and field study of four kinds of truffle (Kamah), *Terfezia* and *Tirmania* species for cultivation. Mushroom Sci X2:507–517

Awameh M, Alsheikh A, Ghawas S (1979) Mycorrhizal synthesis between *Helianthemum ledifolium, H. salicifolium* and four species of *Terfezia* and *Tirmania* using ascospores and mycelial cultures obtained from ascospores germination. Proceedings 4th North American Conference on Mycorrhizae. Fort Collins Colorado, USA

Bokhary HA, Parvez S (1993) Chemical composition of desert truffle *Terfezia claveryi*. J Food Comp Anal 6:285–293

Bokhary HA, Suleiman AAA, Basalah MO, Parvez S (1987) Chemical composition of desert truffles from Saudi Arabia. Can Inst Food Sci Technol J 20:336–341

Bokhary HA, Suleiman AAA, Basalah MO (1989) Fatty acid components of the desert truffle "Al-Kamah" of Saudi Arabia. J Food Protect 52:608

Bratek Z, Jakucs E, Boka K, Szedlay G (1996) Mycorrhizae between black locust (*Robinia pseudoacacia*) and *Terfezia terfezioides*. Mycorrhiza 6:271–274

Cano A, Honrubia M, Molina-Niñirola C (1991) Mycorrhizae in semi-arid ecosystems: synthesis of mycorrhizae between *Terfezia claveryi* Chat., *Picoa juniperi* Vit. and *Helianthemum almeriense* (Cistaceae). 3rd European Symposium on Mycorrhiza Sheffield, UK

Carrillo C, Díaz G, Honrubia M (2004) Improving the production of ectomycorrhizal fungus mycelium in a bioreactor by measuring the ergosterol content. Eng Life Sci 4:43–45

Chang ST, Hayes WA (1978) The biology and cultivation of edible mushrooms. Academic, New York

Chellal A, Lukasova E (1995) Evidence for antibiotics in the two Algerian truffles *Terfezia* and *Tirmania*. Pharmazie 50:228–229

Chevalier G, Dupré C, Riousset L, Dexheimer J (1984) Synthèse mycorhizienne entre *Terfezia leptoderma* Tul et diverses Cistacées. Agronomie 4:210–211

Dexheimer J, Gerardo, Leduc JP, Chevalier G (1985) Étude ultrastructurale comparée des associations symbiotiques mycorhiziennes *Helianthemum salicifolium – Terfezia claveryi* et *Helianthemum salicifolium – Terfezia leptoderma*. Can J Bot 63:582–591

Fortas Z (1990) Etude de trois espèces de Terfez: caractères culturaux et cytologie du mycelium isolé et associé à *Helianthemum guttatum*. Doctoral Thesis, Institut National de la Recherche Agronomique, Clermont-Ferrand

Fortas Z, Chevalier G (1992) Effects des conditions de culture sur la mycorrhization de l'*Helianthemum guttatum* par trois espèces de terfez des genres *Terfezia* et *Tirmania* d'Algerie. Can J Bot 70:2453–2460.

Giovannetti M, Mosse B (1980) An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. New Phytol 84:489–499

Gutiérrez A (2001) Caracterización, micorrización y cultivo en campo de las trufas de desierto. Doctoral Thesis, Universidad de Murcia

Gutiérrez A, Honrubia M, Morte A, Díaz G (1995) Edible fungi adapted to arid and semiarid areas, molecular characterization and in vitro mycorrhization of micropropagated plantlets. Cah Options Mediterr 20:139–144

Gutiérrez A, Morte A, Honrubia M (2001) Ergosterol in desert truffle mycorrhizas. In: Crop and food research (ed) Edible mycorrhizal mushrooms and their cultivation. Christchurch, New Zealand, pp 1–4

Gutiérrez A, Morte A, Honrubia M (2003) Morphological characterization of the mycorrhiza formed by *Helianthemum almeriense* Pau with *Terfezia claveryi* Chatin and *Picoa lefebvrei* (Pat.) Maire. Mycorriza 13:299–307

Honrubia M, Gutiérrez A, Morte A (2001) Desert truffle plantations from South-East Spain. 3rd International Conference on Mycorrhiza, Adelaide, Australia.

Honrubia M, Morte A., Gutiérrez A, González F, Dieste C (2003) Las Turmas o Trufas de Desierto. In: Esteve-Selma MA, Lloréis-Pascual M, Martínez-Gallur C (eds) Los recursos naturales de la región de Murcia. Un Análisis Interdisciplinar. Servicio de Publicaciones de la Universidad de Murcia, Spain, pp 277–279

Honrubia M, Morte A, Gutiérrez A (2005) Six years of the *Terfezia claveryi* cultivation in Murcia (Spain). IV International Workshop on Edible Mycorrhizal Mushrooms, Murcia, Spain, p 70

Janakat SM, Al-Fakhiri SM, Sallal AKJ (2004) A promising peptide antibiotic from *Terfezia claveryi* aqueous extract against *Staphylococcus aureus in vitro*. Phytother Res 18:810–813

Janakat SM, Al-Fakhiri SM, Sallal AKJ (2005) Evolution of antibacterial activity of aqueous and methanolic extracts of the truffle *Terfezia claveryi* against *Pseudomonas aeruginosa*. Saudi Med J 26:952–955

Kagan-Zur V, Raveh E, Lischinsky S, Roth-Bejerano N (1994) Initial association between *Helianthemum* and *Terfezia* is enhanced by low iron in growth medium. New Phytol 127:567–570

Khabar L (1988) Le genre *Terfezia* Tull. (Terfass) de la Forêt de la Mamora (Région de Salé): etude systématique, ecologique, morphologique, cytologique et ultrastructurale. Université Mohammed V, Rabat

Kovacs GM, Vagvolgyi C, Oberwinkler F (2003) *In vitro* interaction of the truffle *Terfezia terfezioides* with *Robinia pseudoacacia* and *Helianthemum ovatum*. Folia Microbiol 48:360–378

Le Tacon F, Jung G, Mugnier J, Michelot P, Mauperin C (1985) Efficiency in a forest nursery of an ectomycorrhizal fungus inoculum produced in a fermenter and entrapped in a polymeric gels. Can J For Res 63:1664–1668

López-González G (1993) Plumbagináceas – Capparaceae. In: Castroviejo S, Aedo C, Cirujano S, Lainz M, Montserrat P, Morales R, Muñoz-Garmendia F, Navarro C, Paiva J, Soriano C (eds) Flora Ibérica. Plantas vasculares de la Península Ibérica e Islas Baleares, vol III. Serv Publ CSIC, Madrid

Marasas WFO, Trappe JM (1973) Notes on southern African Tuberales. Bothalia 11:139–141

Medina I, Satue-Gracia MT, German JB, Frankel EN (1999) Comparison of natural polyphenol antioxidants from extra virgin olive oil with synthetic antioxidants in tuna lipids during thermal oxidation. J Agric Food Chem 47:4873–4879

Moreno G, Galán R, Ortega A (1986) Hypogeous fungi from continental Spain. Cryptogam Mycol. 7:201–229

Morte A, Honrubia M (1992) *In vitro* propagation of *Helianthemum almeriense* Pau (Cistaceae). Agronomie 12:807–809

Morte A, Honrubia M (1994) Método para la micorrización in vitro de plántulas micropropagadas de *Helianthemum* con micelio de *Terfezia claveryi*. Patent n° 9402430. Universidad de Murcia

Morte A, Honrubia, M (1995) Improvement of mycorrhizal synthesis between micropropagated *Helianthemum almeriense* plantlets with *Terfezia claveryi* (desert truffle). In: Elliot TJ (ed) Science and cultivation of edible fungi, vol 2. Balkema, Rotterdam, pp 863–868

Morte A, Honrubia M (1997) Micropropagation of *Helianthemum almeriense*. In: Bajaj YPS (ed), Biotechnology in agriculture and forestry. high-tech and mircropropagation, vol 40. Springer, Heidelberg, pp 163–177

Morte A, Cano A, Honrubia M, Torres P (1994) *In vitro* mycorrhization of micropropagataed *Helianthemun almeriense* plantlets with *Terfezia claveryi* (desert truffle). Agric Sci Finland 3:309–314

Morte A, Lovisolo C, Schubert A (2000) Effect of drought stress on growth and water relations of the mycorrhizal associations *Helianthemum almeriense-Terfezia claveryi*. Mycorrhiza 10:115–119

Morte A, Gutiérrez A, Honrubia M (2004) Desert truffle cultivation: a rehabilitation tool for degraded semiarid lands. In: Faz A, Ortíz R, García G (eds). IV International Conference on Land Degradation, Murcia, Spain

Morte A, Gutiérrez A, Bordallo JJ, Honrubia M (2006) Biotechnology and cultivation of desert truffles: an agricultural tool for degraded semiarid lands. 5th International Conference on Mycorrhiza, Granada, Spain.

Murcia MA, Matínez-Tomé M, Jiménez AM, Vera AM, Honrubia M, Parras P (2002) Antioxidant activity of edible fungi (truffles and mushrooms): losses during industrial processing. J Food Protect 65:22–30

Murcia MA, Martínez-Tomé M, Vera A, Morte A, Gutierrez A, Honrubia M, Jiménez AM (2003) Effect of industrial processing on desert truffles *Terfezia claveryi* and *Picoa juniperi* Vitt: proximate composition an fatty acids. J Sci Food Agric 83:535–541

Phillips IM, Hayman DS (1970) Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc 55:158–161

Roth-Bejerano N, Livne D, Kagan-Zur V (1990) *Helianthemum-Terfezia* relations in different growth media. New Phytol 114:235–238

Rougieux R (1963) Actions antibiotiques et stimulantes de la truffe du desert (*Terfezia boudieri* Chatin). Ann Inst Pasteur 105:315

Taylor FW, Thamage DM, Baker N, Roth-Bejerano N, Kagan-Zur V (1995) Notes on the Kalahari desert truffle, *Terfezia pfeilii*. Mycol Res 99:874–878

Trappe JM, Sundberg WJ (1977) *Terfezia gigantea* (Tuberales) in North America. Mycologia 69:433–437

Wu W, Williams WP, Kunkel ME, Acton JC, Wardlaw FB, Huang Y, Grimes LW (1994) Thermal effects on in vitro protein quality of red kidney bean. J Food Sci 59:1187–1191

Zamora M, Morte A, Gutiérrez A, Honrubia H (2006) *Helianthemum violaceum* Pers., a new host plant for mycorrhizal desert truffle plant production. 5th International Conference on Mycorrhiza, Granada, Spain

Zaretsky M, Kagan-Zur V, Mills D, Roth-Bejerano N (2006) Analysis of mycorrhizal associations formed by *Cistus incanus* transformed root clones with *Terfezia boudieri* isolates. Plant Cell Rep 25:62–70